Plant Physiol. (1968) 43. 238–242

Oxidized and Reduced Nicotinamide Adenine Dinucleotide Phosphate Levels of Plants Hardened and Unhardened against Chilling Injury

Susumu Kuraishi, Noriko Arai, Tadahiro Ushijima, and Tadayoshi Tazaki

Institute of Biology, College of General Education (Kyoyo-gakubu), University of Tokyo. Komaba, Meguro, Tokyo and Department of Plant Protection, Tokyo University of Agriculture and Technology, Fuchu, Tokyo

Received October 31, 1967.

Abstract. **Pea plants (*Pisum sativum* L. var. Alaska) subjected to low temperature (5°) in the light acquired resistance against chilling injury.**

Unhardened plants maintained high NADP and low NADPH levels during illumination at 25° but hardened plants had low NADP and high NADPH levels in the light. When the unhardened plants were transferred to the dark room at 25°, their NADPH levels decreased immediately. On the other hand, hardened plants maintained a high NADPH level for a few hours even in the dark.

Cold injury without ice formation (chilling injury) has been rarely studied (11), although there are many studies of freezing injury (9). The hardening process against freezing injury needs low temperature and light (4). Hardening against chilling injury also might need low temperature and light. If so, the mechanism of frost hardiness proposed by Levitt (5) might be adapted to that of hardening for chilling injury. The SH⇄SS theory is that frost resistance is a resistance towards SH oxidation and SH⇄SS interchange and to formation of intermolecular SS bonds in proteins. Thus the increase in SH content in plants should be observable in the hardened plants, although the increase in protein SH was only observed during the first stage of hardening (3). At lower temperature photosynthetically produced NADPH will be used for other metabolic processes than the fixation of carbon dioxide, due to the markedly decreased CO_2 assimilation. Thus, Levitt (6) assumed that the NADPH produced during the hardening process is available for the protein SS reduction. In this investigation measurement of NADPH and NADP levels in hardened and unhardened plants have been made in order to determine whether the results are consistent with the SH⇄SS theory of hardening against chilling.

Materials and Methods

Seeds of Alaska pea (*Pisum sativum* L. var. Alaska) were sown in pots (15 cm in diameter) in the greenhouse, where the temperature was kept at 25°. Six uniform plants with 8 internodes and of almost the same stem length (approx 30 cm) were selected for the investigation.

Cold Pretreatment (Hardening Process). Every day pea plants were subjected to low temperature (5°) for 3 hours (from 1 PM to 4 PM) either under natural sun light or in complete darkness.

Test for Resistance to Chilling Injury (Survival Test). One day after the last cold-pretreatment pea plants were placed in a large refrigerator at $-3 \pm 1°$ with a fan for 3 hours in the dark and returned to the greenhouse. Five days after the treatment their fresh weights were measured and adopted as a measure of resistance to chilling injury, since plants suffering from chilling injury are killed and show a marked decrease in fresh weight.

Measurements of Fixation of Carbon Dioxide. Fixation of carbon dioxide of the pea plants were measured with an infrared gas analyzer, Hitachi-Horiba EIA-1. Two plants each were cut and placed in an assimilation chamber, with an air flow of 500 ml per minute. The uptake of CO_2 was measured for 10 minutes at 22° under an illumination of 20,000 lux and expressed as mg CO_2 per hour per 100 cm^2 leaf area.

Assay for NADPH and NADP. A preliminary experiment revealed that the levels of NADH and NAD are unchanged after the hardening process. Thus the measurement of nicotinamide nucleotides was limited to NADPH and NADP. A 0.5 g sample of pea plants cut at the third internode was rapidly ground in a glass homogenizer at 90° for 2 minutes in the presence of about 5 times its weight of 0.1 N HCl. The other portion was treated similarly with 0.1 N NaOH. Each was then rapidly transferred to an ice bath, and adjusted to pH 7.6 before 0.3 ml of 0.2 M tris-HCl (pH 7.6) was added. After the total volume of each homog-

enate was measured, it was centrifuged at 10,000 × *g* for 20 minutes at 4°. Immediately after extraction, nicotinamide nucleotide was measured by an assay involving decrease in DCPIP (dichlorophenolindophenol) at 610 mμ using a Hitachi spectrophotometer. Each reaction mixture consisted of 0.7 ml of tissue extract, 0.8 ml of 0.2 M tris-HCl buffer (pH 7.6), 0.1 ml of 0.2 M $MgCl_2$, 0.2 ml and 1.2 mM DCPIP, 0.2 ml of isocitrate dehydrogenase, 0.1 ml of NADPH diaphorase, 0.1 ml of 0.2 μM isocitrate and 0.8 ml of distilled water (13). Recovery of NADP and NADPH added while grinding samples was consistently 85 %. NADPH diaphorase was prepared from spinach chloroplast by the method of Avron and Jagendorf (1), and isocitrate dehydrogenase was prepared from cotyledons of *Vigna sesquipedalis* in the 2nd day of germination by the method of Yamamoto (13). Isocitrate solution was prepared from DL-allo-free isocitric acid lactone (Tokyo Kasei Co. Ltd., Tokyo) as described by Carpenter and Beevers (2).

Results

Relation Between Photosynthesis and the Hardening Process. Pea plants were subjected to cold-pretreatment either in the light or dark, and tested for survival. Fresh weight of control means the fresh weight of the plants kept in a greenhouse without either cold-pretreatment or survival test. Plants cold-pretreated twice both in the light and the dark acquired little resistance to chilling injury, but plants subjected to cold-pretreatment for more than 3 times in the light acquired resistance and gave almost the same fresh weight as that of the control. On the other hand, the plants subjected to cold-pretreatment in the dark showed less resistance than the plants treated in the light. These results suggest that low temperature and light are necessary for hardening against chilling injury. The possibility was considered that plants subjected to cold-pretreatment might have a suppressed utilization of NADPH. If the cold-pretreatment severely suppressed the utilization, the dry weight of the unhardened plants would be expected to be greater than that of the hardened plants. Sixty pea plants were subjected to 5 times cold-pretreatment in the light, and their dry weight was measured. Little difference in the dry weight between unhardened (87.2 mg per plant) and hardened plants (83.2 mg per plant) was found. Moreover, fixation of carbon dioxide measured by an infrared gas analyzer showed only little difference between unhardened (13.7 mg CO_2) and hardened plants (10.9 mg CO_2 per hr per 100 cm^2 leaf area). We might assume that the hardened plants have the ability to utilize NADPH.

Level of NADP and NADPH in Unhardened Plants in the Light. Plants kept in the greenhouse at 25° without cold-pretreatment were harvested from 1 PM to 5 PM at half hour intervals, and assayed for the NADP and NADPH levels (fig 2). Levels of NADP and NADPH was constant during the 4 hour period.

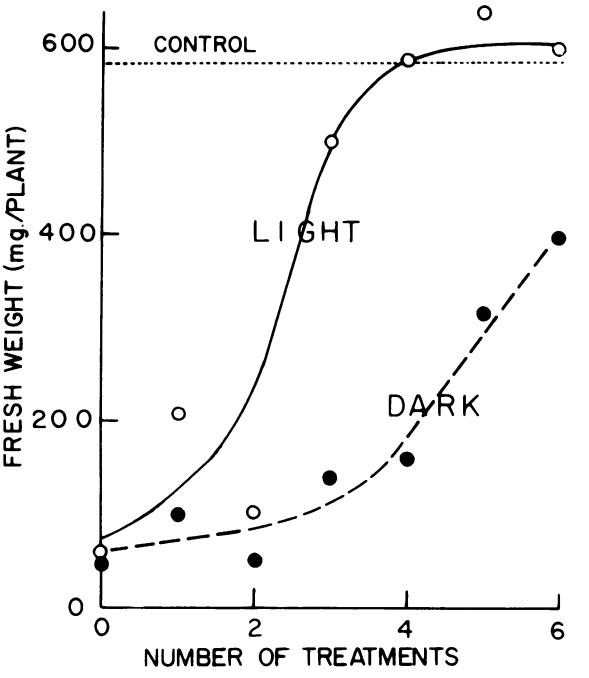
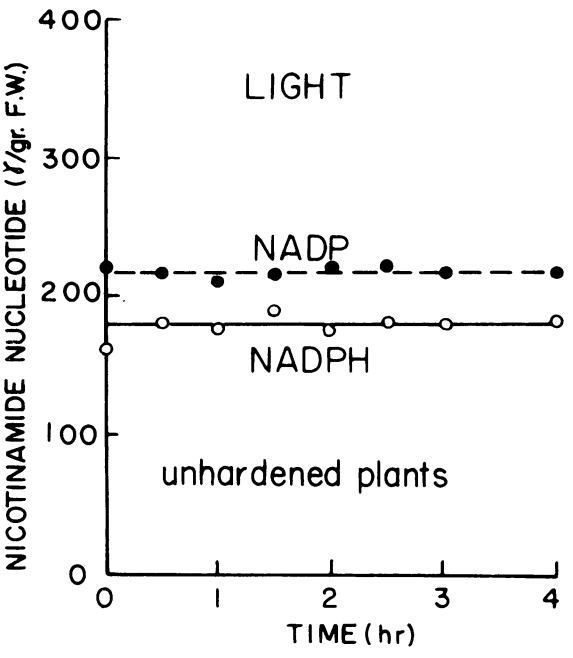
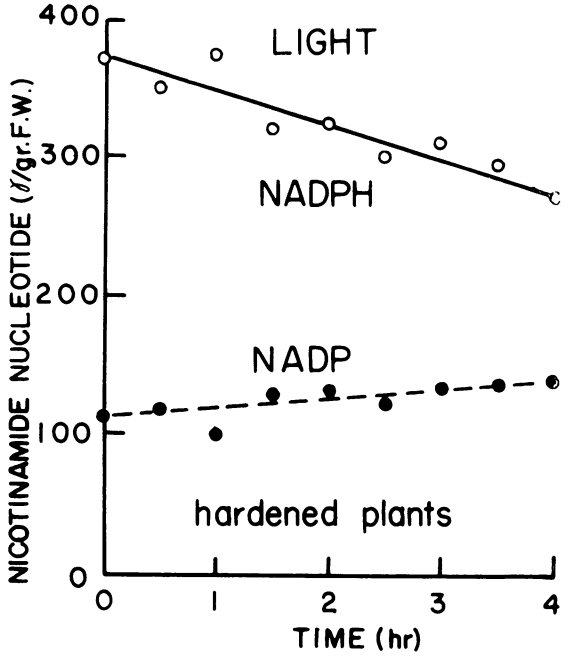


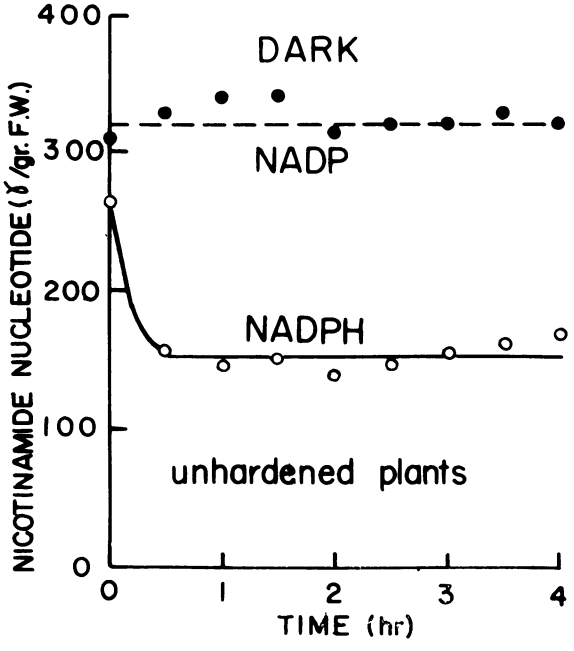
FIG. 1. Hardening under the light or dark. Pea plants were subjected to cold pretreatment either under the light or the dark, and brought to the survival test. Fresh weight of control means the fresh weight of the plants kept in the greenhouse neither cold-pretreatment nor survival test.

Level of Hardened Plants in the Light. A similar experiment was performed using the plants subjected 5 times to a cold-pretreatment. The hardened plants had a higher NADPH than NADP level which is the reverse relation to the unhardened plants (fig 3). The gradually decreased level of NADPH is probably caused by the decrease in sunlight towards evening in the greenhouse. The total amount of NADP and NADPH at any one time is constant, as in the case of unhardened plants.

Level of Hardened and Unhardened Plants after Transfer to the Dark Room. Plants kept in the greenhouse without cold-pretreatment were transferred to the dark room at 25° at 1 PM and assayed for the levels of NADP and NADPH during 4 hours. At the time of transfer to the dark room the levels of NADP and NADPH were similar to the levels in figure 2. Immediately after transfer to the dark room a marked decrease in NADPH level and an increase in NADP level were observed (fig 4). A similar experiment was performed using plants cold-pretreated 5 times (fig 5). A constant decrease in NADPH and a constant increase in NADP levels were observed even within a few hours of the transfer to the dark room, but







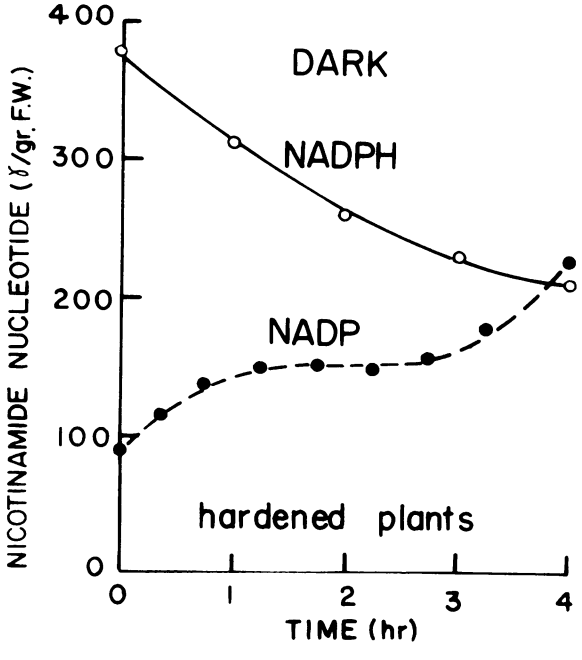


FIG. 2. (Top Left) NADPH and NADP levels of unhardened plants under the light.
FIG. 3. (Top Right) NADPH and NADP levels of hardened plants under the light.
FIG. 4. (Bottom Left) NADPH and NADP levels of unhardened plants in the dark.
FIG. 5. (Bottom Right) NADPH and NADP levels of 5 times cold-pretreated plants (hardened plants) in the dark.

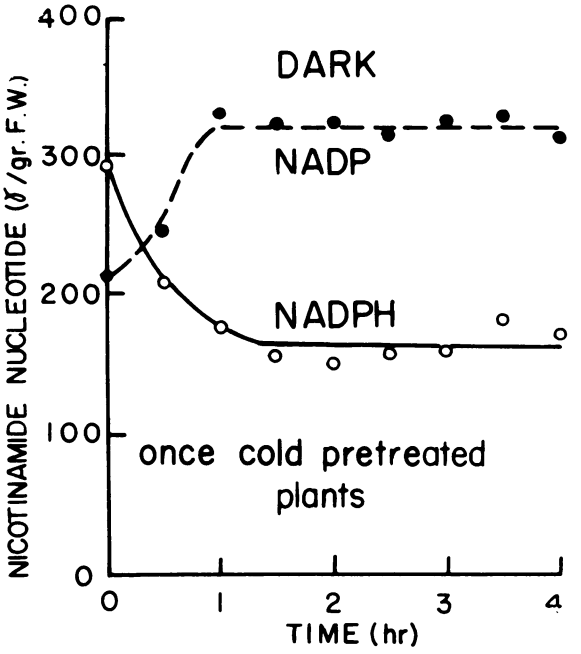


FIG. 6. NADPH and NADP levels of once cold-pretreated plants in the dark.

the velocity of the changes in levels of NADPH and NADP in hardened plants is far slower than that in unhardened plants. The levels of NADPH and NADP were measured using plants cold-pretreated only once (fig 6). Again a decrease in NADPH level and an increase in NADP level were observed. The period, during which NADPH is maintained at a higher level than NADP, however, was longer in the plants that were cold-pretreated 5 times. The periods of time, during which the NADP and NADPH levels have the same value were 220 minutes and 20 minutes in 5 times cold-pretreated and once cold-pretreated plants, respectively.

Discussion

Unhardened pea plants were killed at −3° without ice formation. Most of the studies on freezing injury have been performed under conditions of ice formation in the leaves (9). Thus, the present experiment only deals with chilling injury and not with injury due to ice formation. Frost hardiness takes place at low temperature in the light, and in this experiment hardening against chilling injury also takes place under the same conditions. Such a fact might suggest that the hardiness to freezing and to chilling are caused by the same mechanism. Levitt (5) has emphasized that the removal of intermolecular water to the ice loci plus the compression of the dehydrated protein molecules lead to death of the cell. Unhardened plants subjected to low temperature show sudden opening of stomata and increase in transpiration (11, 12). Thus the removal of intermolecular water from protoplasm and the formation of dehydrated protein in the cells might cause death, as in the case of cold injury with ice formation.

The maintenance of high NADPH level compared to NADP level in the hardened cells clearly supports Levitt's theory that NADPH produced photosynthetically at lower temperature leads to reduction of unknown substances in the cell which may then reduce protein SS sites. If the NADPH formed is maintained at a high level in the chloroplast even in the dark, the dark reaction in photosynthesis should be suppressed. The present experiments clearly show that there is little suppression. But the NADPH in the hardened plants might migrate into the protoplast from the chloroplast. This high level of NADPH in the protoplast might be expected to maintain the high SH content of proteins.

Oh-hama and Miyachi (7, 8), and Orgen and Krogmann (10) observed in *Chlorella* that photosynthetically reduced NADPH disappears completely in a few minutes in the dark. The present study also shows the rapid decrease of NADPH levels in the unhardened plants in the dark. However, hardened plants show a markedly slow decrease of NADPH in the dark. Thus the maintenance of high NADPH levels is an outstanding physiological phenomenon in the hardened plants.

Acknowledgment

The authors express their thanks to Dr. Y. Yamamoto of the Nagoya University for his valuable advice on the measurement of nicotinamide nucleotides. The authors also express their thanks to Dr. J. Levitt of the University of Missouri for reading the manuscript.

Literature Cited

1. AVRON, M. AND A. T. JAGENDORF. 1956. Purification of TPNH diaphorase from chloroplast. Arch. Biochim. Biophys. 65: 475–90.
2. CARPENTER, W. D. AND H. BEEVERS. 1959. Distribution and properties of isocitritase in plants. Plant Physiol. 34: 403–09.
3. KOHN, H. AND J. LEVITT. 1966. Interrelations between photoperiod, frost hardiness and sulfhydryls in cabbage. Plant Physiol. 41: 792–96.
4. LEVITT, J. 1956. Hardiness of plants. Academic Press, New York.
5. LEVITT, J. 1962. A sulfhydril-disulfide hypothesis of frost injury and resistance in plants. J. Theoret. Biol. 3: 355–91.
6. LEVITT, J. 1967. The mechanism of hardening on the basis of the SH⇆SS hypothesis of freezing injury. Cellular injury and resistance in freezing organism. Sapporo. 51–61.
7. OH-HAMA, T. AND S. MIYACHI. 1959. Effects of illumination and oxygen supply upon the levels of pyridine nucleotides in *Chlorella* cells. Biochim. Biophys. Acta 34: 202–10.

8. OH-HAMA, T. AND S. MIYACHI. 1960. Changes in levels of pyridine nucleotides in *Chlorella* cells during the course of photosynthesis. Plant Cell Physiol. 1: 155–62.
9. OLIEN, C. R. 1967. Freezing stresses and survival. Ann. Rev. Plant Physiol. 18: 387–408.
10. ORGEN, W. L. AND D. W. KROGMANN. 1963. Chloroplast nucleotide coenzymes. In: Photosynthetic metabolisms of green plants. B. Kok, ed. National Academy of Sciences, Washington, D. C. 684–87.
11. ONODA, N. 1942. Teion ga shokubutsu ni oyobosu gaisayo no ichi yoshiki ni tsuite (in Japanese). Seitaigaku Kenkyu. 8: 147–69.
12. TAGAWA, T. 1937. The influence of the temperature of the culture water on the water absorption by the root and on the stomatal aperture. J. Fac. Agr. Hokkaido Univ. 39: 271–96.
13. YAMAMOTO, Y. 1963. Pyridine nucleotide content in the higher plant. Effect of age of tissue. Plant Physiol. 38: 45–54.